Effect of Moisture Content on Immobilized Lipase-Catalyzed Triacylglycerol Hydrolysis Under Supercritical Carbon Dioxide Flow in a Tubular Fixed-Bed Reactor

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ABSTRACT: Surplus fats and oils were reacted with several lipases under supercritical fluid conditions for the purpose of obtaining value-added products. Lipases, however, require sufficient moisture content to act as effective biocatalysts. An immobilized lipase from *Candida antarctica* was chosen to examine the rate of enzyme moisture loss under laboratory ambient conditions and also under supercritical fluid conditions. A more important aspect was to determine the effect of lipase moisture content on the hydrolysis of triacylglycerols under the same supercritical fluid conditions. Under ambient conditions at constant air flow, the immobilized lipase lost water at the rate of 4 to 5%/h, from 48.3% moisture to a final moisture content of 0.2%. Water is known not to be very soluble in supercritical carbon dioxide (SC-CO_2). Nevertheless, under supercritical fluid conditions of 60°C, 4000 psi, and carbon dioxide flow rates of 0.5 or 1 L/min measured as expanded gas, the enzyme moisture loss was approximately 2 to 6%/h. To determine the effect of moisture loss on enzymatic hydrolysis, lipase beds in a tubular reactor with moisture contents of 1.5 to 23.5% were reacted with tripalmitin under supercritical conditions. A lipase with an initial moisture content of 1.5% gave little evidence of hydrolysis whereas those containing 5.4 to 23.5% moisture content resulted in products that contained only palmitic acid and unreacted tripalmitin. Thus, optimal parameters for continuous lipase hydrolysis of tripalmitin require: enough enzyme moisture to compensate for complete substrate hydrolysis; sufficient enzyme moisture for losses due to water solubility in SC-CO_2; temperature and pressure sufficient to solubilize the tripalmitin; high carbon dioxide total flow to solubilize all the tripalmitin; and a relatively large enzyme bed volume to increase the solubilized substrate contact time with the enzyme.

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KEY WORDS: *Candida antarctica*, hydrolysis, lipase, moisture effects, supercritical fluid, tripalmitin.

Enzymes as catalysts have been shown to work in nonaqueous solvents. Accordingly, this has become a developing research area that has received much attention in the past 10 yr (1,2). The first reports on the use of supercritical fluids for biochemical reactions also appeared about 10 yr ago (3). Supercritical fluids, especially supercritical carbon dioxide (SC-CO_2), have been investigated not only as extraction solvents but also as reaction media for enzymes. Enzyme reactions in supercritical fluids are seen as potential replacements for chemical catalysts because of mild reaction conditions, substrate specificity, and selectivity. An integrated continuous process of supercritical fluid substrate solubilization, reaction, and downstream separation of product and unreactive substrate without residual solvent is a most efficient protocol.

Many lipases are active in SC-CO_2, and much research has been carried out using lipases in SC-CO_2 to catalyze hydrolysis, esterification, interesterification, and transesterification reactions (1). Consequently, the stability of lipases under high pressure and carbon dioxide is essential in the application of SC-CO_2 to enzyme reactions. Enzyme stability in supercritical fluids is known to depend on pressure, temperature, and enzyme water content (4).

For lipases, catalytic activity is strongly dependent on water content. Therefore, in a continuous high-pressure operation with flowing SC-CO_2, product yield may decrease due to the continuous removal of enzyme moisture by SC-CO_2, resulting in the loss of enzyme activity.

The overall objective of our research group is to develop new processing technologies based upon biocatalysis and supercritical fluids for converting surplus animal fats and vegetable oils into value-added products. One approach is the harvesting of industrially important fatty acids from these fats and oils. The specific objective of this research is to determine the effect of enzyme moisture concentration on the hydrolysis reaction using a model solid triglyceride (tripalmitin). In this study, SC-CO_2 was utilized to solubilize, transport, and extract tripalmitin and its reaction products through a fixed-bed tubular reactor using immobilized lipase containing various levels of moisture. Hydrolysis of tripalmitin was observed under isothermal and isobaric conditions at two SC-CO_2 flow rates.

MATERIALS AND METHODS

Enzyme Novozyme 435 is a thermostable lipase derived from *Candida antarctica* and immobilized on a macroporous

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acrylic resin. Reported activity is 7000 PLU/g. A PLU is a propyl laurate unit formed from 1-propanol and lauric acid in 15 min at 60°C. The enzyme was obtained from Novo Nordisk (Franklintown, NC). Solvents for sample recovery, thin-layer chromatography (TLC), and titration were high-purity, high-performance liquid chromatography (HPLC) grade or reagent grade purchased from Burdick and Jackson (Muskegon, MI), Mallinckrodt (Paris, KY), or J.T. Baker (Phillipsburg, NJ). Deionized water was a laboratory preparation. Carbon dioxide, supercritical fluid chromatography (SFC) SFC grade with dip tube and helium headspace, was purchased from Scott Specialty Gases (Plumsteadville, PA). Polypropylene wool was purchased from Aldrich (Milwaukee, WI). Tripalmitin was from Kodak (Rochester, NY) and palmitic acid (99%+) was from Sigma (St. Louis, MO). Dipalmitin (99%+) and monopalmitin (99%+) were obtained from Nu-Chek-Prep (Elysian, MN). A standard solution of 0.1 N sodium hydroxide was obtained from RED Bird Service (Osgood, IN). A supercritical fluid extractor, model SPE-ED (Applied Separations, Allentown, PA) with a 24 mL stainless steel extraction vessel rated at 10,000 psi (Keystone Scientific, Bellefonte, PA), was used for the supercritical fluid reaction and extraction. Vials (25 mL) from Pierce Chemical (Rockford, IL) were used as traps. TLC plates (5 × 20 cm) of 250 µm Silica Gel G were obtained from Analtech (Newark, DE). HPLC was performed with a Model 114m pump from Beckman Co. (San Ramon, CA), equipped with a reverse phase column of Supelcosil LC 18 from Supelco (Bellefonte, PA), a differential refractometer model R401 from Waters (Milford, MA), an injector model 7125 from Rheodyne (Cotati, CA), and an integrator model 3396 from Hewlett-Packard (Wilmington, DE).

Supercritical fluid reactor. Polypropylene wool, enzyme, and tripalmitin were layered in the stainless steel extraction vessel as shown in Figure 1. Immobilized enzyme (5 g) and tripalmitin (1 g) were weighed on an analytical balance to ±0.1 mg. The extraction vessel, with a thermocouple attached directly to the vessel wall, was placed in the supercritical instrument and brought to temperature equilibrium. Carbon dioxide at 4000 psi and 60°C was allowed to pass through the reactor at flow rates of 0.5 or 1 L/min, measured as expanded gas, for up to 5 h. The temperature of the micrometering valve was 100°C. The extracted lipid fractions were collected in vials at 1 h intervals. The recovered lipid in the vials was removed with chloroform and then the solvent was evaporated on a steam bath under nitrogen flow. Each hourly fraction was then analyzed separately. The combined weight of the fractions was used to calculate percent recovery.

Enzyme drying. Immobilized lipase (5 g) was mixed thoroughly with an equal amount of distilled water in a tared porcelain evaporating dish. The evaporating dish was placed in a hood with a measured air velocity of 247 ft/min. Then, the evaporating dish was weighed periodically to determine the moisture content at various time intervals. Also, immobilized lipases with specific moisture contents were placed in the supercritical instrument and moisture loss determined at

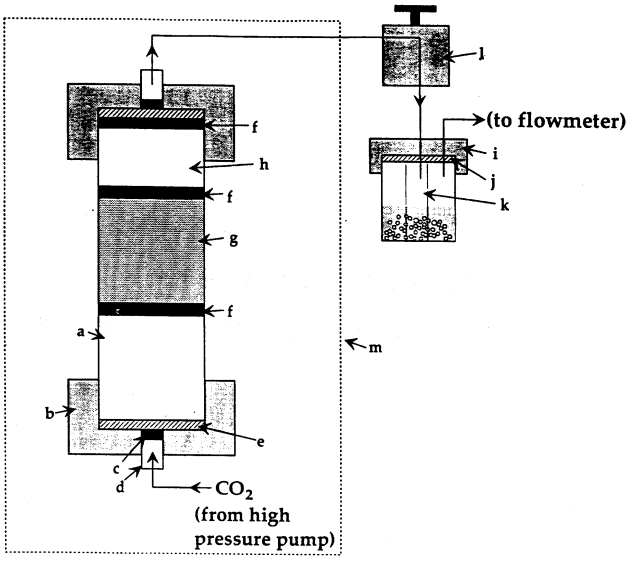

FIG. 1. Schematic of supercritical extractor and trap. Abbreviations: a, stainless steel extraction vessel; b, threaded cap; c, 2 µm frit; d, threaded frit nut; e, peek seal; f, polypropylene wool; g, substrate; h, enzyme; i, vial screw cap; j, vial septum; k, glass tube; l, heated high pressure metering valve; m, constant temperature oven.

4000 psi, 60°C, and two flow rates, 0.5 or 1 L/min measured as expanded gas. Moisture loss was determined by removing the lipase from the supercritical extraction vessel after various amounts of SC-CO_2 were passed through it and then drying the recovered lipase overnight at 120°C to determine residual moisture.

TLC. Thin-layer plates were developed in a glass tank designed for 5 × 20 cm plates. Five µL of samples or standards dissolved in methylene chloride were spotted on the plates and developed with petroleum ether/diethyl ether/acetic acid (80:20:2, vol/vol/vol). Iodine or charring on a hot plate with sulfuric acid were the two methods used to visualize the spots (Fig. 2).

HPLC. Fifty µL of sample were injected into an isocratic mobile phase (50:50:05; acetonitrile/chloroform/acetic acid) at a concentration of approximately 2 mg/mL. Flow rate was 1 mL/min. Attenuation of the refractive index detector was 8×. The integrator was programmed for reduction of noise and elimination of negative peaks in the chromatogram. The analysis took less than 7 min (Fig. 3).

Titration. A weighed sample of product in a 125 mL flask was dissolved in 50 mL of acetone (1% water) on a steam bath. The solution was cooled slightly and titrated with 0.1 N sodium hydroxide solution to a phenolphthalein end point. The amount of palmitic acid present was determined by a comparison with a known weight of palmitic acid standard (99% pure).

RESULTS AND DISCUSSION

The results of lipase hydrolysis of tripalmitin in a tubular fixed-bed reactor are illustrated with the help of the schematic in Figure 1. Carbon dioxide under high pressure (4000 psi)

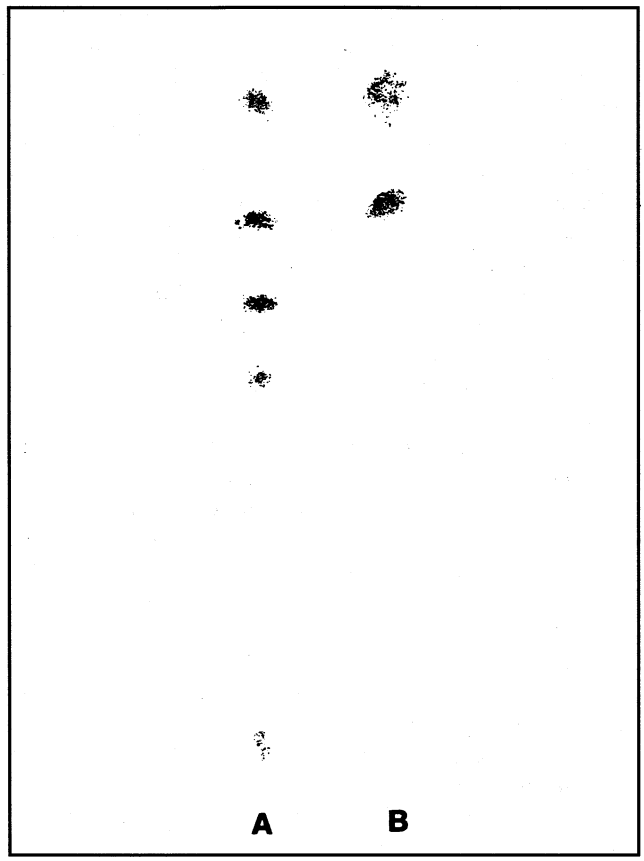

FIG. 2. Thin-layer chromatography analysis. (A) (top to bottom) standards: tripalmitin, palmitic acid, dipalmitin, monopalmitin, plus an impurity at the origin, (B) recovered product. Silica gel G (250 µm), solvent system = petroleum ether/diethyl ether/acetic acid (80:20:2, vol/vol/vol).

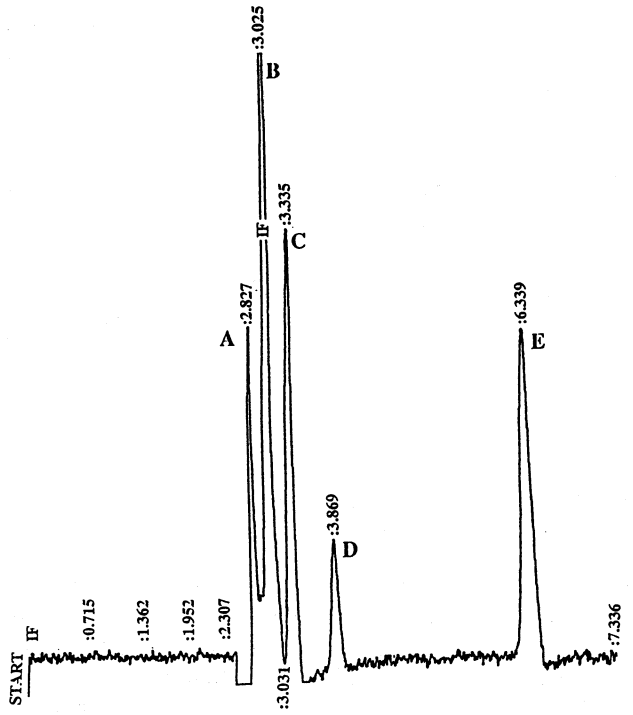

FIG. 3. High-performance liquid chromatography analysis. (A) solvent, (B) monopalmitin, (C) palmitic acid, (D) dipalmitin, (E) tripalmitin. Mobile phase = acetonitrile/chloroform/acetic acid, 50:50:1). Flow = 1 mL/min.

first enters the bottom of the extractor, passes through the substrate bed of tripalmitin (Fig. 1g) and carries solubilized substrate through the enzyme bed (12.4 mL) (Fig. 1h) where lipase hydrolysis occurs. The SC-CO_2, laden with product (palmitic acid) and unreacted tripalmitin, then flows through the heated metering valve at which point the SC-CO_2 is decompressed into the trap. The solids (tripalmitin and palmitic acid) precipitate while the carbon dioxide is vented as a gas. Solid triglycerides and fatty acids like tripalmitin and palmitic acid can be collected either in empty vials or vials partially filled with solvent.

The only products recovered in the trap as determined by TLC were palmitic acid and unreacted tripalmitin (Fig. 2). No dipalmitin, monopalmitin, or glycerol were detected. However, other investigators have found that in the absence of enzyme, the hydrolysis of a triglyceride using moisture-laden SC-CO_2 at high temperature (250°C) and pressure (1160 psi) produces the additional products diglyceride (DG), monoglyceride (MG), and glycerol (5).

Exhaustive solvent extraction of the enzyme bed, after completion of the hydrolysis reaction, and subsequent TLC analysis of the extract from the enzyme bed showed the presence of glycerol as well as dipalmitin and monopalmitin. The glycerol formed during the lipase hydrolysis remained on the enzyme bed because of the known low solubility of glycerol in carbon dioxide (6). Partial glycerides (DG, MG) were not eluted from the reactor at these flow rates. Apparently, the immobilized enzyme bed retains them until complete hydrolysis occurs. With faster flow rates, the partial glycerides might be eluted.

High carbon dioxide pressure (4000 psi) did not have a negative effect on the enzymatic hydrolysis in the present study. However, it has been reported that high pressures may reduce some enzymatic rates (7). Pressures in the range of 4000 ± 1000 psi may not be a critical factor in this particular fixed-bed enzymatic reaction because the solubility of tripalmitin at 60°C does not vary much in that pressure range. Chrastil (8) reported that the solubility of tripalmitin in SC-CO_2 increases only from about 1.5 to 2.0 g/L in the range 3500 to 5000 psi. The temperature (60°C) recommended by the enzyme supplier gave adequate activity, though higher enzyme activity would be expected at 70 to 80°C (9).

To adjust the moisture content of the enzyme prior to packing it into the extractor, the immobilized lipase was completely saturated with an equal weight of water and then dried under ambient conditions to the desired moisture percentage. The surprising ease with which the immobilized lipase lost water is shown in Figure 4. Under ambient conditions (Fig. 4) with an air stream of 247 ft/min, the immobilized lipase

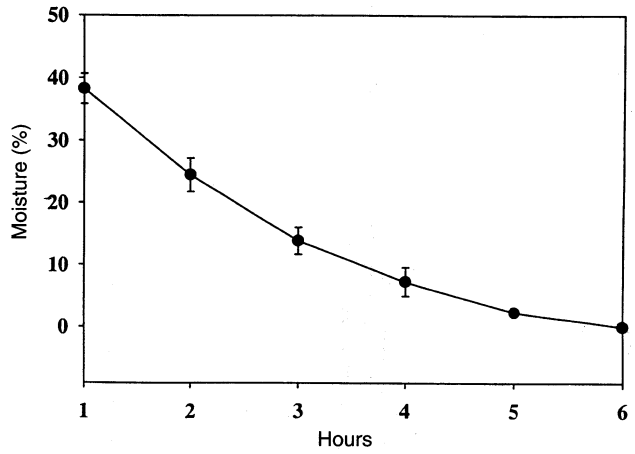

FIG. 4. Enzyme moisture loss at ambient conditions with an air flow velocity of 247 feet per minute.

was totally dehydrated in 6 h, i.e., 48.3% moisture loss. It was thought that the immobilized enzyme (estimated protein, 10%) would retain some moisture at ambient conditions due to the known ability of proteins to hold water.

Under SC-CO_2 conditions (Fig. 5), the amount of moisture loss from the lipase is shown to be a function of the total amount of carbon dioxide passing through the enzyme bed. At 4000 psi and 60°C, it was calculated from Figure 5 that the average solubility of water in SC-CO_2 is 0.271%. For example, Figure 5 shows that when 120 liters of CO_2, approximately 236 g (measured as expanded gas at ambient), were passed through the immobilized enzyme bed, 0.59 g of moisture was removed. Thus, 0.59 g moisture in 236 g CO_2 is 0.25%. The average value compared favorably with the literature value of approximately 0.300% (8,10).

The effect of initial enzyme moisture content on the composition of hourly fractions collected from the lipase-catalyzed hydrolysis of tripalmitin at the SC-CO_2 flow rates (0.5 and 1.0 L/min) is shown in Figure 6. The first fractions recovered are high in palmitic acid but as enzyme moisture is reduced by SC-CO_2, the rate of hydrolysis falls off considerably. One gram of tripalmitin requires 0.067 g of water for complete hydrolysis based on molar calculations. Therefore, a 5-g enzyme bed (5% moisture, i.e., 0.25 g water) should contain sufficient moisture for complete hydrolysis under SC-CO_2 conditions. However, hydrolysis of tripalmitin is incomplete because as found experimentally, in 1 h 60 L of CO_2 removed 0.36 g of moisture from the enzyme as shown in Figure 5.

An HPLC chromatogram of the standards used to analyze the hourly fractions is shown in Figure 3. This method is a modification of a nonpolar reverse phase procedure for separating higher-melting triglycerides from their hydrolysis products (11). Palmitic acid and tripalmitin were the only two compounds observed in the HPLC chromatograms of the products.

The results obtained using tripalmitin as a model solid triglyceride in these lipase-catalyzed hydrolysis studies are listed in Table 1. The initial ratio of substrate to enzyme was 1 to 5. This contrasts with the recommendation for batch operations in nonaqueous solvents to use only 5 to 10% of the weight of immobilized enzyme per substrate (12). Table 1 lists the results of tripalmitin hydrolysis at two SC-CO_2 flow rates. The highest yield of palmitic acid was obtained at the SC-CO_2 flow rate of 1.0 L/min and an enzyme moisture content of 23.5%. Although low enzyme moisture may give poor hydrolysis, too much enzyme moisture is reported to decrease enzyme activity (13,14).

Comparing the results in Table 1 shows that a carbon dioxide flow rate of 0.5 L/min gave better conversions than those

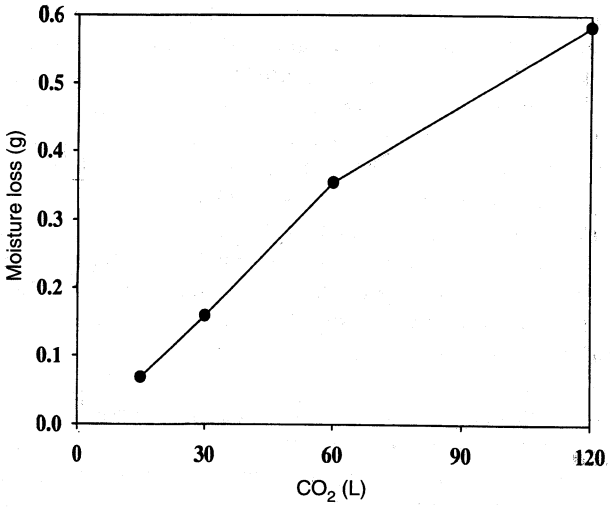

FIG. 5. Enzyme moisture loss (g) under supercritical CO_2 (4000 psi, 60°C) as a function of total CO_2 flow through the immobilized enzyme bed (5 g) in the tubular fixed-bed reactor.

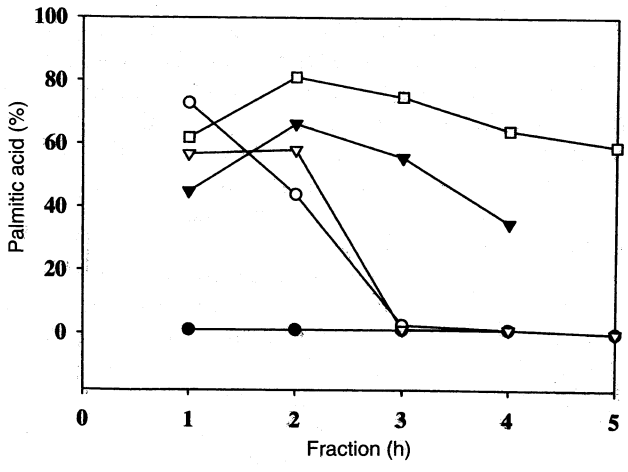

FIG. 6. Palmitic acid in fractions collected under supercritical conditions (4000 psi, 60°C) with lipase of several moisture contents and two carbon dioxide flow rates. ●, 1.5%; ▼, 10.5% moisture (0.5 L/min); ○, 5.4%; ▽, 13.0%; □, 23.5% moisture (1 L/min).

TABLE 1
Product Recovery (based on starting material) and Percentage Palmitic Acid in Product at Two Supercritical Carbon Dioxide Flow Rates and Several Lipase Moisture Percentages

Flow (L/min)[a]	Moisture (%)[b]	Product (g)[c]	Palmitic acid (%)[d]
0.5	1.5	0.300	n.d.[e]
0.5	10.5	0.360	66.2
0.5	23.5	0.489	87.8
1.0	5.4	0.693	18.3
1.0	13.0	0.631	57.5
1.0	23.5	0.893	71.9

[a]Measured as expanded gas.
[b]Initial moisture on 5 g enzyme bed in supercritical flow extractor apparatus.
[c]Hydrolysis product (g) recovered from extractor in 5 h from 1 g tripalmitin starting material.
[d]Palmitic acid (%) in hydrolysis product determined by high-performance liquid chromatography and titration.
[e]n.d. = Not detectable.

obtained at a flow rate of 1.0 L/min. Undoubtedly, this occurred because the substrate, tripalmitin, had more contact time with the enzyme at the lower flow rate. Better total recoveries based on starting material, however, were obtained at the higher flow rate. Obviously, higher flow rates decrease the substrate contact time with the enzyme but increase the total amount of carbon dioxide passing through the extractor in the same time period; therefore, more tripalmitin was solubilized, and more product recovered.

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